University of Alberta

Hybridization of brown lemurs at Berenty Reserve, Madagascar

by

Jan Jekielek

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Hybridization of Brown Lemurs at Berenty Reserve, Madagascar, submitted by Jan Jekielek in partial fulfillment of the requirements for the degree of Master of Science in Systematics and Evolution.

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Abstract

Based on historical data, morphology and genetic compatibility, it appeared that an isolated population of introduced brown lemurs at Berenty Reserve, Madagascar may contain hybrids, *E. f. rufus* X *E. collaris,* and possible backcrosses. Microsatellite DNA fingerprinting was used to study genetic variation and purported hybridization in this population. genetic variation was examined at 10 microsatellite loci for the Berenty population (n=33). Average unbiased heterozygosity was estimated at 76%, and the probability of paternal exclusion was 99.96%. These diversity measures indicate that these genetic markers are suitable for studies of brown lemur parentage and relatedness. As a reference population, a sample of red-fronted brown lemurs at Kirindy Forest, southwestern Madagascar (n=36), was used. At 7 loci, both populations conformed to Hardy-Weinberg equilibrium proportions, indicating random mating, and their levels of genetic variation were similar. Genetic differentiation between the two populations was highly significant ($p<0.0001$); Assignment Test genetic distance (D_{LR}) was 2.27, Nei's standard distance (D_S) was 0.124, and F_{ST} was 0.0438. To assess hybridization in the population, the allele frequencies in the two populations were compared with alleles in pure *E. collaris* from south-eastern Madagascar, and the Berenty population was screened at four microsatellite loci containing diagnostic alleles. At least 30 of 88 Berenty brown lemurs screened proved conclusively to be hybrids, though the actual number is likely to be much higher. For management and captive breeding purposes, all brown lemurs from the Berenty population should be considered hybrids.

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Chapter 1: Introduction

Brown lemurs described

Lemurs, prosimian primates endemic to Madagascar, are ecologically important in all habitats where they occur as primary pollinators of trees (Mittermeier et al. 1994). *Eulemur fulvus*, the brown lemur, is a taxonomic complex that is not as critically endangered as many other lemur taxa. Until recently, brown lemurs were considered a species complex containing 6 geographically separate subspecies (*E. f. fulvus, E. f. rufus, E. f. albifrons, E. f. sanfordi, E. f. collaris,* and *E. f. albocollaris*), occurring in a ring distribution around Madagascar (Mittermeier et al. 1994). Each sub-species is morphologically distinct. It has been contended that *E .fulvus* may be a ring-species (Rumpler 1990), with *E. f. collaris* and *E. f. albocollaris* forming the ends of the ring, since these are the only two subspecies that do not interbreed successfully in captivity (Rumpler 1975). However, *E. fulvus* is likely not a "conventional" ring species, since there are eastern and western populations of *E. f. fulvus* and *E. f. rufus*, suggesting past continuous populations across the Madagascan central plateau in these subspecies (Tattersall 1982). That said, Absher (1994) has suggested that these allopatric populations may represent convergent evolution within each subspecies.

Each subspecies is identifiable by a combination of chromosome numbers, natural range, different pelage, and facial colour patterning, the latter among males in particular (Buettner-Janusch and Hamilton 1979, Hamilton et al. 1980, Mittermeier et al. 1994). However, re-analysis of karyotypic data (Djlelati et al. 1997) and recent molecular phylogenetic analyses of mtDNA sequence data (Wyner et al. 1999a) indicate that *E. f. collaris* and *E. f. albocollaris* are distinct species, and separate from each other and from the four other *E. fulvus* subspecies. For the purposes of this thesis the most recent nomenclature used will be used. Collared brown lemurs will be referred to as *E. collaris*.

E. f. rufus, red-fronted brown lemurs, live in multi-male, multi-female social groups (Sussman 1972, Overdorff et al. 1999). Wild populations of western red-fronted brown lemurs inhabit Analabe Private Reserve and C. F. P. F. Forestry Concession in

south-western Madagascar, locally known as the Kirindy Forest, while eastern *E. f. rufus* exist at Ranomafana National Park (Mittermeier et al. 1994). In 1994, a total of 47 captive red-fronted brown lemurs were housed in 11 North American institutions (Coffman 1994). However, these lemurs represent the offspring of only a few wild-caught animals.

E. collaris, collared brown lemurs, are largely found in southeastern coastal rainforest, but also exist in dryer transitional habitat inland (Mittermeier et al. 1994). Collared brown lemurs can be found at Andohahela Reserve (Mittermeier et al. 1994), and in Ste-Luce Private Reserve (personal observation). In 1994 a total of 43 captive collared brown lemurs were housed in 6 North American institutions (Coffman 1994). As with the red-fronted brown lemurs, these *E. collaris* represent the offspring of a small number of wild-caught animals.

E. f. rufus and *E. collaris* are currently allopatric, though some contact across a river system may have been possible until recently, when deforestation of habitat potentially connecting the two took place (Wyner et al. 2002).

Brown lemur introduction to Berenty Reserve

Berenty Reserve is an 265 ha island of dry gallery forest habitat adjacent to the Mandarare river in south-central Madagascar. At Berenty Reserve, *E. collaris* and western *E. f. rufus* from Analabe Private Reserve were introduced at various times over the past 30 or so years. In the 1930's, a 265 ha section of continuous gallery forest remained undisturbed when a sisal plantation was developed. The peripheries of this forest have subsequently been developed and degraded, and only a slightly smaller portion of this original forest remains. Ringtailed lemurs (*Lemur catta*) and sifaka (*Propithicus verreauxi verreauxi*) are naturally sympatric in this forest.

Around 1974, 8-10 *E. f. rufus* escaped from a cage adjacent to the reserve (J. de Heaulme, personal communication). There are conflicting reports about which of two distinct regions of the reserve was populated first by brown lemurs. Some sources suggest that they entered through the 40 ha northern region of exotic forest (Ankoba), though others say that Malaza, the 90 ha southern region of the reserve was populated first (A.

Jolly, Princeton University, unpublished data). Between 1981 and present, the owner of the reserve introduced 8 - 10 *E. collaris* of unknown sex into the reserve (J. de Heaulme, personal communication). There was a single male collared brown at the reserve as early as 1966 (A. Jolly, unpublished data), though it is unknown if this individual was alive when the red-fronted browns arrived.

In 1990, animals were noticed in the population that exhibited a phenotype intermediate to *E. f. rufus* and *E. collaris* (J. Davidson, unpublished data). It was hypothesized that such animals could be hybrids between the two species, since they are known to hybridize in captivity. Figure 1 summarizes the population growth of the Berenty brown lemur population over the past 20 years, with reference to the numbers of *E. fulvus*. In 1996, several hybrid infants were apparent in Berenty (S. Pinkus, UBC, unpublished data). Anaramalangy, a 110 ha region of degraded forest continuous with Malaza, was first censused in 1995. The minimum population estimate for *Eulemur fulvus* in this region was 8 individuals.

Kaleta Reserve is a 150 ha section of forest semi-continuous with Berenty's Anaramalangy. Although there have been attempts to develop Kaleta to compete for tourism with Berenty, the former was heavily grazed by cattle. Thus the habitat is highly disturbed, and is unlikely to support large populations of lemurs. Although a cattle passage separates the two reserves, it is possible for lemurs to move between them. At least two collared brown lemurs were introduced to Kaleta within the past ten years, as well as at least one red-fronted brown. Four brown lemurs living as a troop were found in this area in 1996 (S. Pinkus, UBC, unpublished data), and they are believed reserve guides to be the offspring of a female *E. collaris* and a male *E. f. rufus*.

Why study hybridization at Berenty Reserve?

The introduction and potential hybridization of brown lemurs into Berenty Reserve makes it possible that the population has undergone an extreme founder effect. It is hypothesized that the founding group was comprised of few individuals, with little subsequent immigration. Assuming that the two species did not interbreed, one might

expect each sympatric population to have started with low genetic variation, with the loss of variation consequently enhanced by genetic drift.

In brown lemurs, mating between close relatives in captivity results in high infant mortality relative to many mammalian species (Ralls et al. 1988). Furthermore, despite the fact that adult males are sometimes related to adult females in their troop, mating pairs are rarely related (Merenlender 1993). This suggests that some form of inbreeding avoidance mechanism, outside of male dispersal, may be present in *E. f. rufus*. Both these sources of evidence pointed towards a noticeable cost of inbreeding in this species (Gandon 1999).

However, since the monitoring of the Berenty brown lemurs began, their population has been growing at a high rate (Figure 1). If *E. collaris* and *E. f. rufus* interbred, it is possible that this mixture of genes provided a release (Keller et al. 2001) to the cost of inbreeding for the population. The collared brown lemurs may have provided the variability needed to offset the inbreeding depression that is likely associated with the founding population of *E. f. rufus*

There have been concerns about animals of hybrid origin being introduced into natural populations of *E. fulvus*. It has also been suggested that in order to protect the naturally occurring populations of ringtailed lemurs (*L. catta*) from potential competitive interactions with *E. f. rufus* (S. Pinkus, UBC, unpublished), it may be necessary to remove or transplant the latter population (A. Jolly, unpublished). If it is decided that the Berenty brown lemurs should be transplanted, definitive identification of hybrids may help reserve managers in deciding what options are available with reference to maintaining the genetic integrity of natural *E. f. rufus* and *E .collaris* populations.

Finally, brown lemur hybridization had only until recently been assessed in a lab setting, using karyotyping as a tool. Given that it is not often possible to use invasive sampling techniques to obtain blood samples, and that lab facilities are uncommon in Madagascar, it was necessary to develop a simple, reliable assay that would require minimal sampling conducted in a non-invasive fashion. Molecular genetic techniques are ideal, since genetic samples in the form of hair or feces could be used. Microsatellite genetic markers were chosen as a tool, since a few had already been developed for brown

lemurs (Merenlender, 1993), and they allowed simple assessment of genetic variation within and between populations.

Hybridization among lemur taxa

Hybridization among lemurs has been a subject of study among lemur geneticists because of the significant karyotypic differences among many lemur taxa. In a captive setting, there have been many studies on intra and inter-species hybridization, particularly in the genus *Eulemur* (Dutrillaux and Rumpler 1977, Hamilton and Moses 1979, Moses et al. 1979, Rumpler and Dutrillaux 1980, Ratomponirina et al. 1982, Rumpler et al. 1984, Rumpler et al. 1985, Warter and Rumpler 1985, Sakanakura and Kondo 1987, Rumpler et al. 1990, Djlelati et al. 1997). Hybridization has been proposed as a major force in the speciation of the *Lepilemur* genus (Ishak et al. 1992) as well as in *Eulemur* (Jung 1997, 2000).

That said, very few hybrid zones have been documented among lemurs in the wild. These few include a hybrid zone among black lemurs (*Eulemur macaco*) (Rabarivola et al. 1991), and brown lemurs (*E. fulvus*) in two different regions, Betsakafandrika (Lehman and Wright 2000) and Andringitra (Wyner et al. 2002), and small groups of black X brown lemurs where the two species exist sympatrically (Y. Rumpler, personal communication). A hybrid zone has only been quantified genetically by Wyner et. al. (2002) for *E.f.rufus* X *E. albocollaris*. To examine hybridization, they found private and diagnostic sites, specific to populations and species, respectively, in several regions of brown lemur DNA. These sites were identified using population aggregation analysis (PAA), and then used to screen the hybrid zone. The regions examined were one mitochondrial region (d-loop), three nuclear introns (hemopexin, malic enzyme, ceruloplasmin), and the flanking region of the Efr26 microsatellite that was developed as part of this thesis.

Development and use of microsatellites in lemur species

Microsatellites were first isolated in lemurs as early as 1993 (Merenlender 1993), the year of a landmark study that used them to study paternity exclusion, community

structure, and phylogeography in wild chimpanzees (Morin et al. 1993). However, to these loci were not used in any other published study of lemurs until this research was begun. A human microsatellite was characterized in *E. fulvus* in 1994, but it was found to be monomorphic (Calvas et al. 1994). Unfortunately, it was possible to amplify only three of the 9 loci described in the Merenlender study, not enough to look at a representative portion of the genetic variation. As a result, it was necessary to develop a new microsatellite DNA fingerprinting system for brown lemurs (Jekielek and Strobeck 1999). Twelve microsatellites were found that yielded easily quantifiable genotypes, and it was possible to use them along with the three previously mentioned loci to assess genetic variation at Berenty reserve. Some of the microsatellites exhibited variation in other taxa in the family Lemuridae (see Addendum).

More recently, use of microsatellites in lemur population genetics has blossomed. In 2000, they were used in fat-tailed dwarf lemurs (*Cheirogaleus medius*) to help determine mating strategy, identifying high rates of extra-pair copulation (Fietz et al. 2000). In 2001, microsatellites were characterized and tested for Verreaux's sifaka (*Propithecus verreauxi verreauxi*) (Lawler et al. 2001), as well as for the grey mouse lemur, *Microcebus murinus* (Radespiel et al. 2001a). The latter loci were used to determine detailed social structure in grey mouse lemurs, allowing for testing of kin selection hypotheses (Radespiel et al. 2001b), as well as tests of paternity in captive populations (Andres et al. 2001, Radespiel et al. 2002). In 2002, one microsatellite locus (Efr26), which was used to assess hybridization in this thesis, was used to study hybridization between *E. f. rufus* and *E.f.albocollaris* in a brown lemur contact zone at Andringitra National Park (Wyner et al. 2002). Later in 2002, 14 microsatellites were characterized and tested in the grey bamboo lemur (*Hapalemur griseus*) (Sommer et al. 2002).

Apart from using microsatellites, studies of lemur population genetics and genetic assessment of parentage have been conducted in black lemurs (*Eulemur macaco*) using blood proteins (Rabarivola et al. 1996), RAPDs (Rabarivola 1998, Fausser et al. 2000), and combinations of these methods (Rabarivola et al. 1998). Such genetic analyses were suggested as a first step in breeding programs (Scheffrahn et al. 1998). In the *Lepilemur* genus, blood proteins were used to assess genetic diversity and structure in red-tailed

sportive lemurs, *Lepilemur mustelinus ruficaudatus* (Tomiuk et al. 1997). Later, RAPDs were used to assess genetic differentiation among populations of this species (Bachmann et al. 2000), as well as among populations of *Lepilemur septentrionalis* and *Lepilemur dorsalis* (Ravaoarimanana et al. 2001). RAPDs have also been used to determine genetic diversity and structure in wild (Hafen et al. 1998) and captive (Neveu et al. 1998) populations of grey mouse lemur (*Microcebus murinus*). Acoustic and RAPD-genetic differentiation were found to correlate in *M.murinus*, and were used to identify distinct colonies of this species. Further, RAPDs were utilized for paternity studies among groups of *E. fulvus* (Gachot-Neveu et al. 1999). Finally, within-species mitochondrial DNA polymorphism was used to aid in captive breeding and reintroduction efforts for black and white ruffed lemurs, *Varecia variegata variegata* (Wyner et al. 1999b).

In summary, field-based population genetic analyses of lemurs began using blood protein polymorphisms, with RAPDs coming in shortly thereafter, a method that persisted until recently. Although microsatellites for lemurs were developed as early as 1993, they have only come to be regularly used in lemur populations only recently, as of 2000. They are becoming the method of choice for genetic characterization of lemurs.

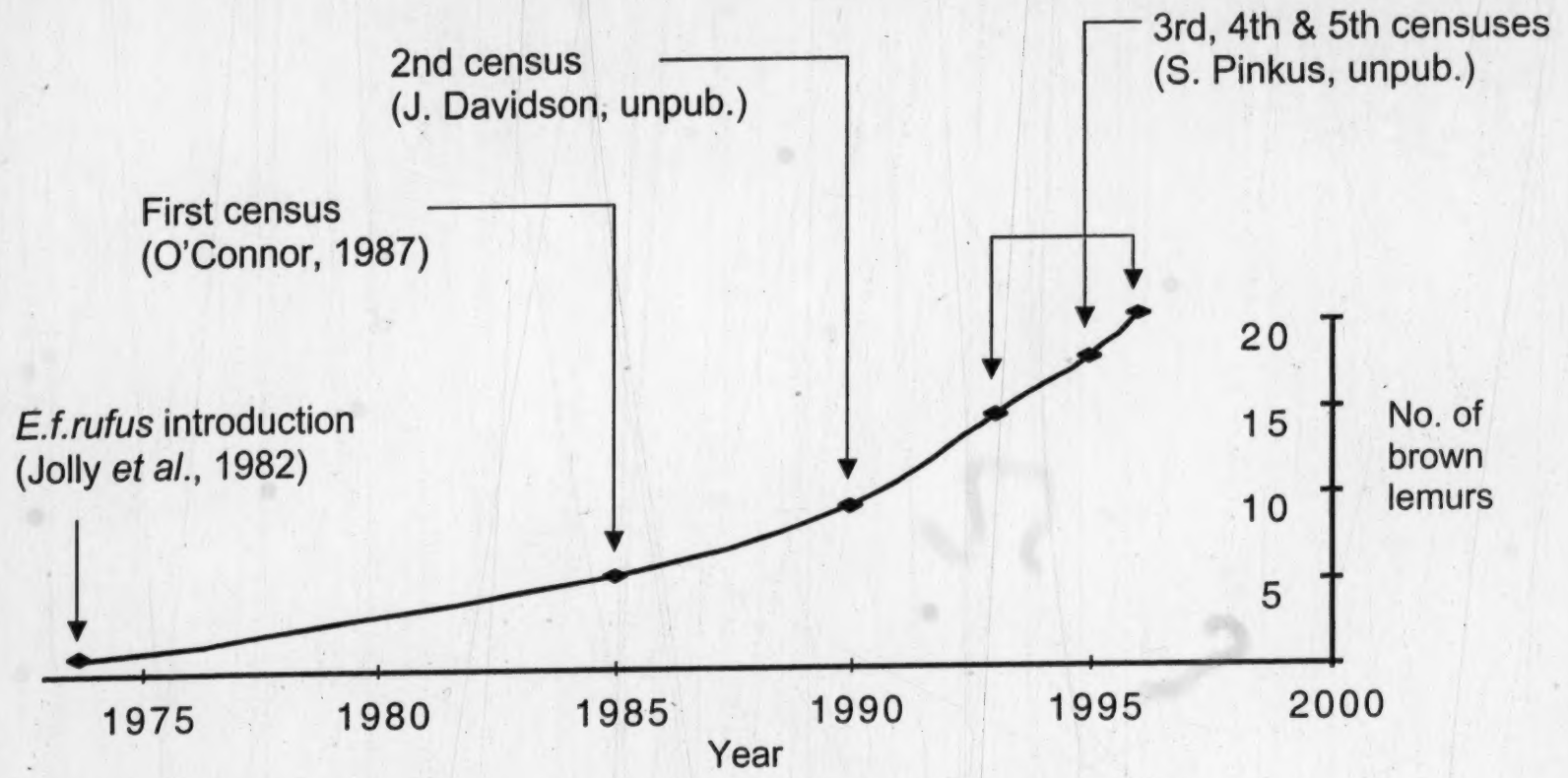

Figure 1-1: Population history of brown lemurs at Berenty Reserve, Madagascar.

E. f. rufus were introduced in 1974; the two documented *E. collaris* introductions were prior to 1980. Lemurs exhibiting morphology intermediate to the two subspecies were first documented in the 1990 census.

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Chapter 2: Genetic variation and hybridization in a population of introduced brown lemurs (*Eulemur fulvus*) at Berenty Reserve, Madagascar.

Abstract

Based on historical data, morphology and genetic compatibility, it appeared that an isolated population of introduced brown lemurs at Berenty Reserve, Madagascar may contain hybrids, *E. f. rufus* X *E. collaris*, and possible backcrosses. Microsatellite DNA fingerprinting was used to study genetic variation and purported hybridization in this population. genetic variation was examined at 10 microsatellite loci for the Berenty population (n=33). Average unbiased heterozygosity was estimated at 76%, and the probability of paternal exclusion was 99.96%. These diversity measures indicate that these genetic markers are suitable for studies of brown lemur parentage and relatedness. As a reference population, a sample of red-fronted brown lemurs at Kirindy Forest, southwestern Madagascar (n=36), was used. At 7 loci, both populations conformed to Hardy-Weinberg equilibrium proportions, indicating random mating within each population, and their levels of genetic variation were similar. Genetic differentiation between the two populations was highly significant ($p<0.0001$); Assignment Test genetic distance (D_{LR}) was 2.27, Nei's standard distance (D_S) was 0.124, and F_{ST} was 0.0438. To assess hybridization in the population, the allele frequencies in the two populations were compared with alleles in pure *E. collaris* from south-eastern Madagascar, and the Berenty population was screened at four microsatellite loci containing diagnostic alleles. At least 30 of 88 Berenty brown lemurs screened proved conclusively to be hybrids, though the actual number is likely to be much higher. For management and captive breeding purposes, all brown lemurs from the Berenty population should be considered hybrids.

Introduction

Most of the primate fauna on the island of Madagascar is considered threatened as a result of human influences, and is listed on Appendix 1 of CITES and in Class A of the African Convention (Harcourt and Thornback 1990). Deforestation and habitat fragmentation are increasing (Mittermeier et al. 1994), confining many species to isolated pockets of habitat, and limiting their ability to disperse. Berenty Reserve, comprised of 265 ha of spiny and gallery forest in southeastern Madagascar, is one such habitat fragment, populated by 5 naturally occurring species of lemurs (Harcourt and Thornback 1990). At least two introduced brown lemur species, *Eulemur fulvus rufus* and *Eulemur collaris*, also reside at Berenty Reserve.

Brown lemur introductions to Berenty Reserve: Implications

Over the last 25 years, two naturally allopatric species of brown lemur have been introduced into the reserve: about 8 red-fronted lemurs (*E. f. rufus*) from Analabe Private Reserve, western Madagascar in 1974, and at least two collared lemurs (*E. collaris)* prior to 1980 (Jolly et al. 1982). The approximate ranges of these two species are found in Figure 1. It is possible, but undocumented, that other brown lemurs were introduced there between that time and the present. Although collared lemurs have recently been elevated to full species status based on mtDNA sequence data (Wyner et al. 1999, Wyner et al. 2002) and chromosomal studies (Djlelati et al. 1997), the two are known to interbreed and produce fertile offspring in captivity (Rumpler 1975). The reproductive costs or benefits of such hybridization do not appear to have been documented quantitatively in the wild.

Early in the history of the introduction, at the St. Cathrine's Workshop (1996), it was suggested that the few collared lemurs introduced to Berenty be removed, to prevent hybridization with *E. f. rufus*. In 1990, lemurs with pelage and facial morphology intermediate to *E. f. rufus* and *E. collaris* were documented in the population (J.

Davidson, unpublished data). J. Davidson subsequently hypothesized that such animals could be hybrids between the two species.

Knowledge of the existence and the extent of red-fronted X collared lemur hybridization is of considerable importance to future lemur research and management at Berenty Reserve. Of particular note is the fact that brown lemurs are competing for resources with the native *Lemur catta* (S. Pinkus, UBC, unpublished data). If brown lemurs are found to have a negative demographic effect on *L.catta* or on forest regeneration, it may be proposed that they be removed from the reserve in the interest of conservation of the native species. Since it would be inappropriate to place hybrids in natural populations of *E. f. rufus*, knowledge of hybridization would help define the removal options.

Identification of hybrids

The considerable variation in individual pelage and facial colouration among brown lemurs (Harcourt and Thornback 1990) precludes assaying hybridization purely on the basis of individual appearance. Although hybridization can be assessed in lemurs by means of karyotyping (Rumpler 1975, Djlelati et al. 1997), this method requires invasive (blood) sampling and can be costly and time consuming. Conversely, when using molecular genetic methods that utilize Polymerase Chain Reaction (PCR) to amplify DNA, genetic samples can be gathered non-invasively, in the form of hair follicles (Morin et al. 1994) or feces (Gerloff et al. 1995). Hybridization between taxa has been quantified using such molecular methods, via determination of variable mitochondrial DNA haplotypes (Abernathy 1994) and microsatellite genotypes (Garcia-Moreno et al. 1996) of putative hybrids. In both these cases, the presence of diagnostic markers (markers otherwise found exclusively in unmixed populations of one of the species) from one species in the other was used as an indicator of hybridization.

The utility of mitochondrial haplotypes in detecting hybridization is limited in mammals because mitochondrial DNA is usually inherited only through matrilines (Hedrick and Miller 1992), whereas microsatellites are inherited in a codominant

Mendelian fashion. This makes it possible to determine hybridization of females of one taxon with males of the other, and vice versa. Microsatellites have been shown repeatedly to be a good means of quantifying genetic variation within and among populations (Paetkau et al. 1997, Radespiel et al. 2001, Whitehouse and Harley 2001). In this study, evidence of hybridization is presented, and genetic variation in the brown lemur population at Berenty Reserve is assessed and compared to other populations of brown lemurs, using microsatellites developed in brown lemurs (Merenlender 1993, Jekielek and Strobeck 1999).

Methods

Sample collection and putative hybrid identification

Between September and December, 1996, a census of the brown lemur (*Eulemur fulvus*) population at Berenty Reserve, Madagascar, was conducted using methods designed specifically for this are (S. Pinkus, UBC, unpublished). Troops of lemurs were found and counted, and distinctive marks were placed on two or three individuals per troop by means of a black hair-dye. These and natural distinguishing features of unique individuals were used to identify troops. Troops were recounted several times to verify troop size. This process, facilitated by the small size of the reserve, was repeated until no new troops were found. Individuals that appeared to be collared lemurs (*Eulemur collaris*) or potential hybrids (*E. f. rufus* X *E. collaris* or possible backcrosses) were identified based on a simplistic assay of pelage and facial colouration (See Appendix 1).

Many of the lemurs at Berenty are highly habituated to human presence. This allowed for direct collection of genetic samples in the form of hair follicles. Animals were lured with bananas and a few hairs were manually plucked, usually from the tips of their tails. This did not appear to disturb the animals at all.

In the fall of 1997, a second series of samples at Berenty Reserve was collected by Vodra and Sabine, students at the University of Antananarivo. In the summer of 1998, a series of pure-strain *E. f. rufus* samples were collected at the C. F. P. F. Forestry

Concession in south-western Madagascar (S 20° 03', E 44°39'), locally known as Kirindy. Kirindy is one of three reserves which comprise the Menabe Forest, which includes Analabe and another reserve, Andranomena (Zinner et al. 2001). Since Analabe and Kirindy are in the same stretch of forest and in close proximity to one another, it seemed reasonable to use the Kirindy brown lemur population as representative of the Berenty source population. In the fall of 1999, through a collaborative agreement with Barbara Wimmer of the Ludwig Maximillians University of Munich, it was possible to examine additional pure red-fronted brown lemur samples from Kirindy. In addition, genetic samples from several pure *E. f. rufus* were obtained from the Tsimbazaza Zoo in Antananarivo, Madagascar, and the Assiniboine Park Zoo, Winnipeg, Canada.

Genetic samples from collared lemurs external to Berenty were gathered from habituated animals in Fort Dauphin, Madagascar. Also, samples from wild animals in the Manangotry region of south-eastern Madagascar were gathered by Tsirovelo Ratovoson. The approximate locations of all of the sampling areas are found in Figure 1.

Genetic analyses

Genetic variation was examined in the first sample of the Berenty population (n=33) at 14 microsatellite loci: Efr02, Efr05, Efr08, Efr09, Efr24, Efr26, Efr30, Efr37, Efr56, Efr81 and Efr88 (Jekielek and Strobeck 1999), and I3, L1 and L2 (Merenlender 1993). Variation was also quantified at these loci for six collared lemurs and nine red-fronted lemurs from other, unmixed populations. All loci were amplified, and individual genotypes determined as in Jekielek and Strobeck (1999). Due to restrictions in time and amount of genetic material, and reservations about certain loci (see the results section), genotypes were obtained at seven loci (Efr08, Efr24, Efr30, Efr37, Efr56, Efr81 and Efr88) for the second Berenty sample (n=25) and the Kirindy population (n=36). Genetic variation measures within and between populations were calculated predominantly using GENEPOP (Raymond and Rousset 1995), but also using Microsoft Excel.

For the first Berenty sample, number of alleles per locus, observed heterozygosity (H_O), expected heterozygosity under Hardy-Weinberg proportions [H_e, as in Nei and

Roychoudhury (1974)], and probability of paternal exclusion (P_{pe}), the probability that a random male in the population can be excluded as a potential father, were calculated. The divergence of each locus from Hardy-Weinberg proportions was quantified using the Exact Test (Guo and Thompson 1992). Overall, these genetic diversity measures, as well as genetic distances, Nei's Standard Distance (D_S), and Assignment Test Distance (D_{LR}) (Paetkau et al. 1997), were compared between the Berenty and Kirindy populations. To compare allele frequencies in these two populations, and to examine hybridization at the population level, an Assignment Test (Paetkau et al. 1995, Paetkau et al. 1997) was conducted.

Sequence variation at locus Efr26 was examined in the Berenty population (n=21), and in 6 collared and 9 red-fronted lemurs from populations external to Berenty. This microsatellite was amplified as in Jekielek and Strobeck (1999), with the following differences: volumes of all reagents except template DNA were scaled up to 100 µl, and both primers in each reaction were unlabelled. PCR products were gel extracted via QIAquick™ spin columns (QIAGEN), and sequenced on an ABI 377 DNA Sequencer using a dRhodamine Terminator Cycle Sequencing kit (Perkin-Elmer). Allele sequences were first determined in homozygotes (Berenty population n=8; external *E. collaris* n=3, *E. f. rufus* n=6). For heterozygotes (Berenty population n=13; external *E. collaris* n=3, *E. f. rufus* n=3), the sequence of each allele was inferred based on alleles found in homozygotes. Sequences were aligned using Sequence Navigator™ software (Applied Biosystems), and a maximum parsimony phylogeny of alleles was constructed using PAUP 3.1 (Swofford 1993).

Results

Sample collection and putative hybrid identification

Whereas most of the 202 lemurs found during the 1996 Berenty Reserve census appeared to be red-fronted lemurs, 20 individuals had the morphological appearance of putative hybrids. Only one collared lemur was found, but it was not possible to obtain a sample. Hair samples were collected from 33 brown lemurs, of which nine were possible hybrids. In the second Berenty sample, hair was collected from 62 individuals, six of which were judged as prospective hybrids. Comparing between each sample, only three individuals had identical genotypes. From unmixed populations external to Berenty, six pure collared lemur samples were obtained. Nine pure samples of pure *E. f. rufus* were obtained initially from zoos, followed by a total of 46 samples from Kirindy Reserve.

Genetic analyses

Measures of microsatellite variation for the first sample of the Berenty brown lemur population at all loci except for Efr26 are given in Table 1. Locus Efr26 was excluded from this analysis because its size variation was the result of a nucleotide insertion in DNA flanking the microsatellite repeat region (Table 2). All loci conformed to Hardy-Weinberg equilibrium proportions, except Efr02, Efr09, and L1 (Exact Test, $p<0.001$, Table 1). Examination of these three non-conforming loci reveals large discrepancies between expected and observed heterozygosities, of at least 50% each. The presence of this considerable excess of homozygosity in these but not the other loci indicated the possible presence of null alleles, potentially biasing results (Callen et al. 1993). Thus, these three loci were excluded from further analysis of overall variation in the population.

At the remaining loci, within-population genetic variation measures for Berenty and Kirindy brown lemur populations are shown in Table 3. Neither population deviated

significantly from Hardy-Weinberg equilibrium at any of the loci (X^2 test, overall $p<0.58$). For genetic differentiation measures between the Berenty and Kirindy populations, Assignment Test genetic distance (D_{LR}) had a value of 2.27, Nei's standard distance (D_S) had a value of 0.124, and F_{ST} had a value of 0.0438; their genetic was differentiation highly significant at $p<0.0001$, G-test (Goudet et al. 1996).

Of the microsatellite loci examined, four appeared to have alleles diagnostic of *E. collaris*: Efr81, Efr37, I3, and Efr26. These loci are summarized in Table 4. In collared brown lemurs, locus Efr81 was monomorphic for allele 182 (n=6). Allele 182 was found only in one individual of *E. f. rufus* (n=52), from an allopatric eastern population of this species. In the external *E. f. rufus* populations, Locus Efr37 had all odd-numbered alleles (221, 223, etc.). Even-numbered alleles (232, 234) were found only in *E. collaris*. At locus I3, alleles in the range of 141bp – 146bp were diagnostic of collared lemurs (n=6), and alleles 115bp – 140bp were diagnostic of red-fronted lemurs (n=11). For all of three of these loci, alleles indicative of both *E. collaris* and *E. f. rufus* status were found in the Berenty population.

At locus Efr26, there was no variation among individuals in the microsatellite itself, but significant variation in sequence was found in the region flanking it. Ten nucleotide positions were found to be variable, and sites diagnostic between the two species were found at three of these (Table 2). Alleles R1-R8 were diagnostic of red-fronted lemurs, and alleles C1-C2 were diagnostic of collared lemurs (Table 4). All of 48 most parsimonious unrooted phylogenetic trees derived from the sequence variation among these alleles supported a phylogenetic separation between collared lemur (C) alleles and red-fronted lemur (R) alleles (Figure 2). Both R and C alleles were found in a sample of the Berenty population (Table 4).

At the population level, the results of the Assignment Test between Berenty and Kirindy populations, including six collared lemurs from pure *E. collaris* populations, are shown in Figure 3. More unlikely genotypes occur further up and right from the origin along the diagonal.

Hybridization assay

When considering the phylogenetic diagnostic marker locus Efr26 alone, 11 of 21 individuals tested were were found to be hybrids, due to the presence of C alleles (Figure 4). Two of these individuals were homozygotes for collared lemur alleles. Four of nine individuals that were suspected hybrids based on morphology were found to lack C alleles.

When considering all available lines of evidence concerning hybridization, including all diagnostic loci and morphological indicators, at least 30 animals among the total of 88 sampled at Berenty were found to be hybrids. Thirty is a minimum estimate because it was not possible to test all individuals at all four available lines of genetic evidence. Most of the hybrids (63%) were diagnosed based on only one of the five available lines of evidence, while only three of them were heterozygotes at all diagnostic loci (Figure 5). These three individuals were morphologically hybrids; there were also two individuals not assessed at locus Efr26 that were heterozygotes for *E. collaris* alleles at the remaining three loci, one of which was hybrid based on physical appearance. Two of the individuals that were heterozygotes at all loci were also the most habituated and obviously aggressive animals in the reserve (S. Pinkus, UBC, unpublished data).

At the population level, at 7 microsatellite loci, all pure-strain *E. collaris* individuals tested (n=6) had allele frequencies that were more similar to those of the Berenty population than to those of the Kirindy population [Assignment Test, (Paetkau et al. 1995), Figure 3].

Discussion

Validity of microsatellite genotypes obtained

When the first sample of the Berenty population is considered, 10 of 13 microsatellite loci conformed to Hardy-Weinberg expectations; the other three exhibited large relative heterozygote deficits. Although it is possible that the relatively high

homozygosity at these loci is due to an evolutionary mechanism such as heterozygote disadvantage, a more plausible explanation is that there are non-amplifying (null) alleles at these loci, since such alleles are common among microsatellites (Callen et al. 1993, Paetkau and Strobeck 1995). The only way to test this would be to redesign the primers that amplify the suspect loci, and rescreen the population. These three heterozygote-deficient loci may in fact follow the same patterns as the majority of the loci. However, these three loci were not necessary to conduct a reasonably accurate assessment of genetic variation in the brown lemur populations considered in this study, and so were excluded from the analysis.

Within population variation

Values of genetic variation (heterozygosity, average number of alleles per locus) in the first Berenty sample were similar when 10 and 7 microsatellite loci were used, indicating that results based on 7 loci were just as accurate as results based on 10. Genotypes from both the first and the second Berenty sample were pooled to include as much of the variation at the 7 loci as possible. Neither the Berenty nor the Kirindy brown lemur populations deviated significantly from Hardy-Weinberg equilibrium at any of the loci considered, and the corresponding inbreeding coefficient (F_{IS}) was negligible. This implies that both populations exhibit near random mating, and have little or no selection happening within them, at least at the loci in question. Previous studies have not examined brown lemur population structure from a population genetic perspective. Parentage and life history studies conducted in eastern brown lemur populations indicate that females are largely philopatric, while males disperse into other troops upon becoming adults (Merenlender 1993, Overdorff et al. 1999). Further, brown lemurs are known to differ in behavioural patterns among different habitats, particularly with respect to their resource partitioning (Tattersall and Sussman 1998), and western brown lemur populations exist in dramatically different (wet vs. dry forest, respectively) habitats (Tattersall 1982). It is unclear if the social structure is the same in eastern and western brown lemur populations, or what effect their sex-differential dispersal pattern has on the

genetic sub-structure of brown lemur populations. The effect of the uncharacteristically high density of lemurs at Berenty reserve, as well as the effect of resource competition of brown lemurs with the native *L.catta* (S. Pinkus, UBC, unpublished data) on their behaviour is also unclear.

When comparing genetic variation within Berenty and Kirindy populations, the Kirindy heterozygosity is only slightly higher, as is the average number of alleles per locus. Assuming that Kirindy values are representative of eastern brown lemur populations, the within-population genetic diversity in the Berenty population has likely not decreased greatly from that of its original, source *E. f. rufus* population. These levels of variation may be the result of randomly high variability among the introduced *E. fulvus*, the introduction of more brown lemurs than has been documented, the addition of *E. collaris* alleles to the gene pool, the recentness of the introduction, or most likely a combination of these factors. However, the maintenance of this level of observed heterozygosity in the Berenty population supports the idea that the lemurs are mating randomly.

The high values of probability of paternal exclusion (P_{pe}) in both populations establish the suitability of the ten loci used in this paper to assess variation for molecular genetic studies of brown lemur relatedness, parentage and individuality, in populations with similar levels of genetic diversity. By showing relatedness and individual relative reproductive success within populations, such data can complement behavioural studies. For instance, in the (Pereira and McGlynn 1997) study of *E. f. rufus* affiliative behaviour, microsatellites could aid in clarifying the fitness tradeoffs made by pair-bonded males, something nearly impossible to address from behavioural data alone.

Between population variation

When comparing the Berenty and the Kirindy brown lemur populations, genetic differentiation between the two populations was highly significant. The introduction of 8 or even a few more brown lemurs would likely not have transferred a representative

sample of the genetic variation to the Berenty population, leading to a pronounced founder effect and consequent genetic differentiation..

When comparing the Berenty and Kirindy populations, the latter has double the private alleles that the Berenty population has (Table 3), and likely as a result of this has a larger spread in its probabilities of genotypes (Figure 3). Two of the private alleles in the Berenty population, Efr37 allele 232 and Efr81 allele 182, apparently came from *E. collaris*; the remaining three were found in the most variable loci in the population. It is possible that these latter alleles are rare, and so do not exist among the Kirindy animals that were sampled, or that the actual source population of the Berenty animals differed from Kirindy in allelic composition.

This is the only study to date study to use microsatellite DNA fingerprinting techniques to assess genetic variation in lemurs. The only other microsatellite study to date looked at parentage (Merenlender 1993). Other markers such as RAPDs and blood proteins, which have been used to assess variation in related black lemurs (Rabarivola et al. 1996, Rabarivola et al. 1998, Scheffrahn et al. 1998, Fausser et al. 2000), are likely to have different levels of genetic variation inherent to them (Rabarivola et al. 1998, Balloux et al. 2000). Given that microsatellites have the advantage of being highly repeatable relative to RAPDs (Perez et al. 1998), and considerably more variable than blood proteins, using these markers for future population genetic and relatedness studies in brown lemurs is recommended.

Hybridization assay

The DNA based hybridization assay described in this paper can identify individuals that are hybrid *E. f. rufus* X *E. collaris*. However, due to segregation and independent assortment in the nuclear genome, it cannot establish with certainty that animals testing negative, or lacking diagnostic alleles, are a pure strain of either species. The assay is biased towards recent hybridization; introgression that occurred further in the past is more difficult to diagnose with each successive generation, due to segregation and independent assortment in the nuclear genome. This is highlighted by several

individuals in the population of brown lemurs at Berenty Reserve that appear to be hybrids based on morphology, but which lack diagnostic alleles. The examination of further loci that exhibit variation diagnostic of each species, both nuclear and the maternally-inherited mitochondrial genome [as in Wyner et al. (2002)], will increase the power of assessing such hybridization.

Four microsatellite loci with alleles diagnostic of *E. collaris* were used for the hybridization assay. Of these, Efr26 provides by far the strongest evidence of hybridization, due to the strong phylogenetic separation of alleles from *E. f. rufus* and from *E. collaris*. In Efr26, while there is no variation among individuals in the microsatellite sequence itself, there are many variant sites in the flanking region. The three independently evolving nucleotide sites at locus Efr26 that phylogenetically separate alleles originating in collared lemurs from those originating in red-fronted ones provide strong evidence that suites of alleles in each species have been evolving separately for some time. This locus can thus be used effectively for hybridization diagnosis, as well as to provide independent nuclear characters for phylogenetic analyses of *E. fulvus*.

At locus Efr81, the diagnostic allele 182 was found to be fixed in *E. collaris* , and present in only one individual pure red-fronted brown lemur (n=52). However, this individual was from an eastern population, one that has likely been allopatric from the source population of the Berenty animals for hundreds of years (Tattersall 1982). It is likely that this allele does not occur in western populations of red-fronted brown lemurs.

At locus Efr37, even-numbered alleles were found at high frequency in *E. collaris*, while *E. f. rufus* had exclusively odd-numbered alleles (n=54). For the purposes of this study, the presence of the even-numbered allele 232 in the Berenty population indicates hybrid status. However, some odd-numbered alleles may have passed into the Berenty population from the introduced *E. collaris* , resulting in an underestimation of hybridization.

At locus I3, smaller alleles (115bp – 140bp) were diagnostic of red-fronted brown lemur status, and larger ones (141bp – 146bp) were diagnostic of collared brown lemurs. These *E. collaris* alleles exist at a low frequency in the Berenty population.

For these three latter microsatellite loci that were not sequenced, there is less certainty that the alleles are actually diagnostic. The sample sizes of pure *E. collaris* in all cases, and of pure *E. f. rufus* in the case of Locus I3, are small for a microsatellite-based analysis. It is unclear in each case whether the mutation distinguishing the set of alleles in one species from the other is in the microsatellite, or at different positions in the flanking region, as it is in Efr26 and alleles of chimpanzee microsatellites sequenced by (Garza et al. 1995). This distinction is significant due to the high mutation rate (and the correspondingly high probability that two alleles could be not identical-by-descent) commonly associated with microsatellites (Jarne and Lagoda 1996), but not with random sequences. It has been demonstrated that after significant amounts of evolutionary time, microsatellites no longer provide an effective diagnostic tool (Paetkau et al. 1997). Screening a larger population of *E.f.collaris* , and other *E. f. rufus* populations, as well as screening the diagnostic microsatellite loci sequence variation among individuals in these populations, will provide greater certainty for the use of these loci for diagnostic purposes.

Although it would require invasive (blood) sampling, an excellent way to assay for hybridization and to test the robustness of this molecular genetic assay would be through karyotypic analysis. Since *E. f. rufus* have have a diploid chromosome number of 60, and *E. collaris* have a diploid chromosome number ranging between 50 and 52, Berenty hybrids would be expected to have an intermediate number of chromosomes (Djlelati et al. 1997). Using this technique, it may be possible to determine the extent of hybridization of individual animals, based on their deviation in number of chromosomes from each species in its pure form.

Extent of hybridization in the population

By using only locus Efr26 to examine hybridization in the Berenty brown lemur population, 11 out of 21 animals studied were diagnosed as hybrids. When considering all diagnostic loci and morphological indicators, at least 30 animals among the total of 88 sampled at Berenty were found to be hybrids. However, the actual number is likely to be

much greater than this, as several factors pointing to this being an underestimation. Firstly, it was not possible to test all individuals for all lines of genetic evidence, and secondly, this hybridization assay likely missed some hybrid animals for the reasons described in the previous section. Most of the hybrids that were diagnosed were done so based on only one of the five available lines of evidence (Figure 5).

Because this was a study of microsatellite genetic variation in both the Berenty and the Kirindy populations, it was possible to evaluate hybridization based on comparison of population allele frequencies. At 7 microsatellite loci, all pure-strain *E. collaris* individuals (n=6) grouped many orders of magnitude more closely with the Berenty population. That said, the position of the *E. collaris* individuals largely far away from the majority of the Berenty animals on the graph indicates that collared lemur genotypes as a whole are unlikely to occur in the Berenty population, due to the presence of private alleles in *E. collaris*. This suggests that only a few of the allelic variants at these 7 loci existing in natural *E. collaris* populations have made it into the Berenty population.

In the Berenty population, there were both individuals lacking diagnostic alleles that had a distinct hybrid appearance, and individuals that were definitely hybrids genetically that did not look so based on their morphology (Figure 4). Considering the morphologically distinct lemurs lacking diagnostic alleles, it is possible that these individuals fit into natural variation patterns in *E. f. rufus*, or more likely that hybridization is persistent, and happened early during the introduction, with the alleles used for diagnosis having been selected out, or just absent by sampling limitations. The genetically obvious hybrids that do not appear to be morphologically so are likewise probably the products of introgressive hybridization. For example, this is highlighted by occurrence of two individuals that are homozygotes for collared lemur (C) alleles at locus Efr26. These two individuals, neither of which are collared lemurs based on morphology, could only have been the offspring of either an *E. collaris* and an introgressant or of two introgressants, indicating that hybrids remain fertile past the first generation of hybridization. Such fertility of hybrids has been seen documented in *E. f. rufus* and *E. collaris* hybrids in laboratory studies, having no apparent reproductive cost (Rumpler 1975). The persistence of *E. collaris* genes in the Berenty population suggests strongly

that hybridization happened early in the history of the population, and that at least there is no cost, if not a reproductive benefit, to cross-breeding. Any reproductive costs or benefits of such hybridization have yet to be carefully documented.

Why might hybridization be commonplace in the Berenty Reserve brown lemur population? It is possible, but not measurable using these techniques, that *E. collaris* genes provide a slight selective advantage to the individuals possessing them in the Berenty population. Two of the first generation hybrid individuals are large, highly habituated and aggressive, and have feeding priority in their troops (S. Pinkus, UBC, unpublished data), and data is not available for other hybrids. There have been several studies of animals (deer, grey seals, pilot whales, guppies, albatrosses) where populations retain more of their genetic variation than expected based on random mating, due to increased mating among more outbred individuals (Amos and Balmford 2001).

Another possibility is that hybridization with *E. collaris* has aided the introduced *E. f. rufus* in overcoming inbreeding depression. Although this may not be the case in natural populations, in captivity brown lemurs were found to have a high cost of inbreeding, or a high number of lethal equivalents (Ralls et al. 1988). Repeated introductions of *E. collaris* into the Berenty red-fronted lemur population may have resulted in higher levels of heterozygosity in the mixed population than might be expected if the populations of the two species were separate, allowing for successful breeding and population growth. Although the long-term viability of this population remains uncertain due to its small size, the limited availability of its resources, and the tentative nature of its welcome at Berenty, at present the population continues to increase. The Berenty brown lemurs might be considered an anthropogenically created incipient hybrid species.

The extent and timing of hybridization in the Berenty population can be further elucidated through use of a greater number of diagnostic nuclear loci, sequencing of mitochondrial haplotypes diagnostic of brown lemur species (Absher 1994, Wyner et al. 2002), and genotyping of greater proportion of individuals in the population.

Conservation and research-related implications

The two species of brown lemur hybridizing at Berenty Reserve occur naturally in allopatric distributions, and existed separately for a sufficient amount of evolutionary time to allow for phylogenetic differentiation (Absher 1994, Wyner et al. 1999). It is unlikely that these species would ever have had the opportunity to hybridize in the wild. For management and captive breeding purposes, all brown lemurs from the Berenty population should be considered hybrids. It has been recommended to the owners of the reserve that the animals not be moved to other regions of Madagascar (S. Pinkus, UBC, unpublished final report of the 1998 *E. fulvus* census at Berenty Reserve). If the animals are moved from the reserve into captivity, they should be kept separate from pure-strain *E. f. rufus* and *E. collaris*, to maintain the genetic integrity and diversity of captive brown lemur populations. Hybridization between species has augmented the genetic diversity of genetically depauperate taxa such as the Florida panther (Obrien et al. 1990), but this type of captive breeding strategy is not warranted here since significant numbers of brown lemurs still exist in the wild, and have considerable genetic diversity, as this study demonstrates.

Red-fronted lemur behaviour has been studied at Berenty Reserve (Pitts 1995, Kaufman 1996, Caless 1998), and in the latter reference were considered to be *E. f. rufus*. However, hybridization may have resulted in atypical behaviour relative to natural unmixed populations. For instance, collared lemurs may have different vocalizations than red-fronted ones (R. Kitko, pers. comm.), and vocalizations of hybrids may differ from those of either species. Care should be taken in all future research to recognize the Berenty Reserve brown lemur population as being composed of hybrid animals.

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Table 2-1: Berenty Brown Lemur First Sample Statistics

Number of alleles per locus, expected heterozygosity under Hardy-Weinberg proportions (He), observed heterozygosity (Ho), and percent difference between Ho and He, for 13 microsatellite loci in the first sample of the brown lemur population at Berenty, Madagascar (n=33). For underlined loci, Ho is significantly different from He (P<0.001; Exact Test, Guo and Thompson, 1992).

Locus	Number of Alleles	He	Ho	Ho % Difference	Exact Test p-value
Efr02	3	44.9%	21.2%	- 52.8%	0.000
Efr05	9	73.5%	81.8%	+ 11.3%	0.751
Efr08	13	83.9%	75.7%	- 9.8%	0.233
Efr09	6	78.8%	39.3%	- 50.1%	0.000
Efr24	8	81.4%	69.6%	- 14.5%	0.426
Efr30	5	63.7%	60.6%	- 4.9%	0.700
Efr37	8	76.0%	78.7%	+ 3.6%	0.170
Efr56	5	70.4%	72.7%	+ 3.2%	0.132
Efr81	5	76.1%	63.6%	- 16.5%	0.006
Efr88	5	70.3%	81.8%	+ 16.4%	0.906
I3	8	84.8%	84.8%	0%	0.858
L1	5	48.3%	21.2%	- 56.1%	0.000
L2	9	80.6%	78.7%	- 2.3%	0.523

Table 2-2: The 90 base-pair region of variation in locus Efr26, for all allelic variants.

Underlined nucleotides are the 10 positions exhibiting variation. Positions 4, 25 and 74 are diagnostic between *E. f. rufus* and *E. collaris*. Presence or absence of a C nucleotide at position 3 determines size variation at this locus in the Berenty population. The microsatellite is found between positions 64-72.

Allele	Sequence
C1	CCCGTCTAAC CTCCACGGAA AAAA-GACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCACGTGTG
C2	CC-GTCTAAC CTCCACCGAA AAAA-GACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCACGTGTG
R1	CC-ATCTAAC CTCCACCGAA AAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTC TGCGCGTGTG
R2	CC-ATCTAAC CTCCACCGAA AAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCGCGTGTG
R3	CC-ATCTAAC CTCCACCGAA AAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCGTGTGTG
R4	CC-ATCTAAC CTCCACCGAA GAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTC TGCGCGTGTG
R5	CC-ATCTAAC CTCCACCGAA GAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCGCGTGTG
R6	CC-ATCTAAC CTCCACCGAA GAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCGTGTGTG
R7	CC-A-CTAAC CTCCACCGAA AAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGTG TGCGCGTGTG
R8	CC-ATCTAAC CTCCACCGAA AAAAAGACAC TATTCATCAA CATACTGGAT TTGTGTATGT GGCGTGTGT- -GCGCGTGTG

Table 2-3: Within population genetic variation measures for Berenty and Kirindy brown lemur populations.

Loci refers to the number of loci genotyped per individual, # Alleles is the average number of alleles per locus, and # Private is the total number of alleles across 7 loci that are private to each population relative to the other. H_e and H_o are the unbiased expected and observed heterozygosities, respectively, and F_{IS} is the inbreeding coefficient. P_{pe} is the probability of paternal exclusion, the probability that a random male in the population can be excluded as a potential father.

Population	# Loci	n	# Alleles	# Private	H_e	H_o	F_{is}	P_{pe}
Berenty 1996 sample	10	33	7.3	-	75.90%	74.50%	0.018	99.96%
Berenty both samples	7	56	7.0	5	75.68%	73.94%	0.023	99.62%
Kirindy (Forêt-Suisse)	7	36	8.0	10	76.40%	76.56%	-0.002	99.75%

Table 2-4: Alleles found at four diagnostic loci in independent *E. collaris* and *E. f. rufus*, and Berenty populations.

Numbers denote the number of individuals that have at least one copy of a given allele. At locus Efr26 sequence variation has been quantified; all 10 variant positions are presented with the rest of the sequence excluded (see Table 2). Only size variation has been examined in the other loci.

Locus	Allele Name	Distinguishing Molecular Variation	External *E. collaris*	External *E. f. rufus*	Berenty Population
Efr26		Sequence	(n=6)	(n=9)	(n=21)
	R1	-ATCAACTGC	-	-	2
	R2	-ATCAAGTGC	-	7	8
	R3	-ATCAAGTGT	-	-	4
	R4	-ATCGACTGC	-	-	1
	R5	-ATCGAGTGC	-	3	5
	R6	-ATCGAGTGT	-	-	1
	R7	-A-CAAGTGC	-	1	-
	R8	-ATCAA--GC	-	1	-
	C1	CGTGA-GTAC	5	-	8
	C2	-GTCA-GTAC	4	-	3
I3		Size	(n=6)	(n=11)	(n=48)
	115	115bp	-	1	-
	124	124bp	-	1	-
	128	128bp	-	2	12
	130	130bp	-	2	13
	132	132bp	-	2	16
	134	134bp	-	7	22
	136	136bp	-	3	8
	138	138bp	-	1	-
	140	140bp	-	1	-
	141	141bp	3	-	3
	142	142bp	3	-	-
	144	144bp	4	-	6
	146	146bp	1	-	7

Table 2-4 continued

Locus	Allele Name	Distinguishing Molecular Variation	External *E. collaris*	External *E. f. rufus*	Berenty Population
Efr81		Size	(n=6)	(n=52)	(n=60)
	182	182bp	6	1	7
	186	186bp	-	42	26
	188	188bp	-	21	31
	190	190bp	-	15	24
	192	192bp	-	3	-
	194	194bp	-	2	-
	196	196bp	-	7	12
Efr37		Size	(n=6)	(n=54)	(n=88)
	219	219bp	-	-	25
	221	221bp	-	11	15
	223	223bp	-	30	10
	225	225bp	1	13	44
	227	227bp	-	10	45
	229	229bp	-	6	-
	231	231bp	-	17	8
	232	232bp	5	-	9
	233	233bp	1	7	-
	234	234bp	3	-	-
	235	235bp	-	2	5
	237	237bp	-	1	-
	239	239bp	-	1	-

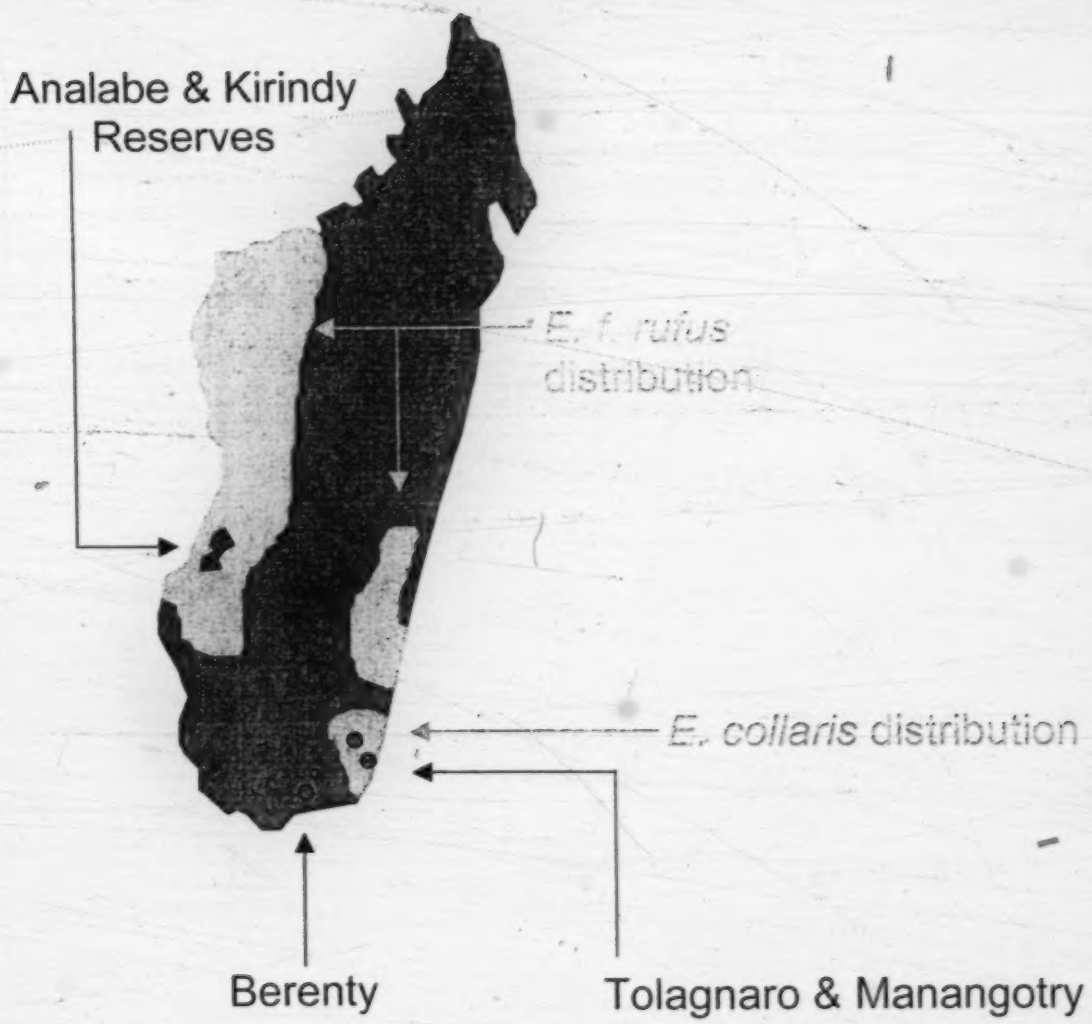

Figure 2-1: Red-fronted and collared brown lemur distributions, and approximate sampling locations.

In western Madagascar, Analabe Reserve, the actual source population of the Berenty red-fronted brown lemurs, is the northern parcel, continuous with the more southern Kirindy (C. F. P. F. Forestry Concession). In eastern Madagascar, Tolagnaro is coastal, and Manangotry is further inland. This figure was adapter from Mittermeier *et. al.* (1994).

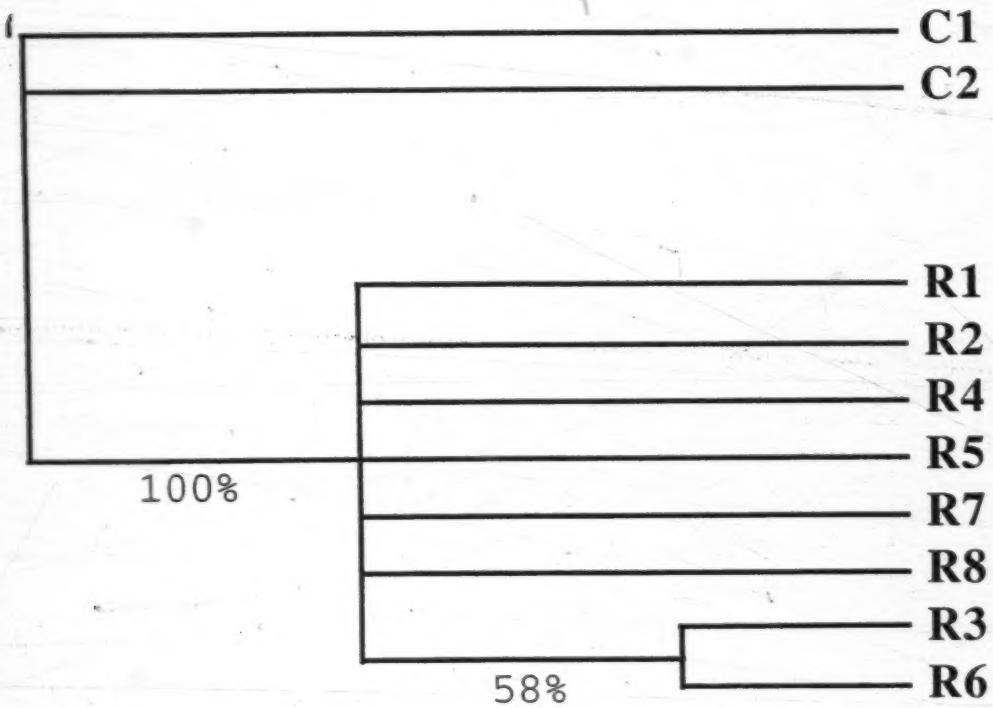

Figure 2-2: The 50% majority rule consensus tree (of 48 most parsimonious unrooted trees) for the 10 allelic variants at locus Efr26.

At the top of the figure are alleles with diagnostic variation for *E. collaris* (C alleles); at the bottom are diagnostic alleles for *E. f. rufus* (R alleles). The percentage of most parsimonious trees that share a common node is given beside each branch.

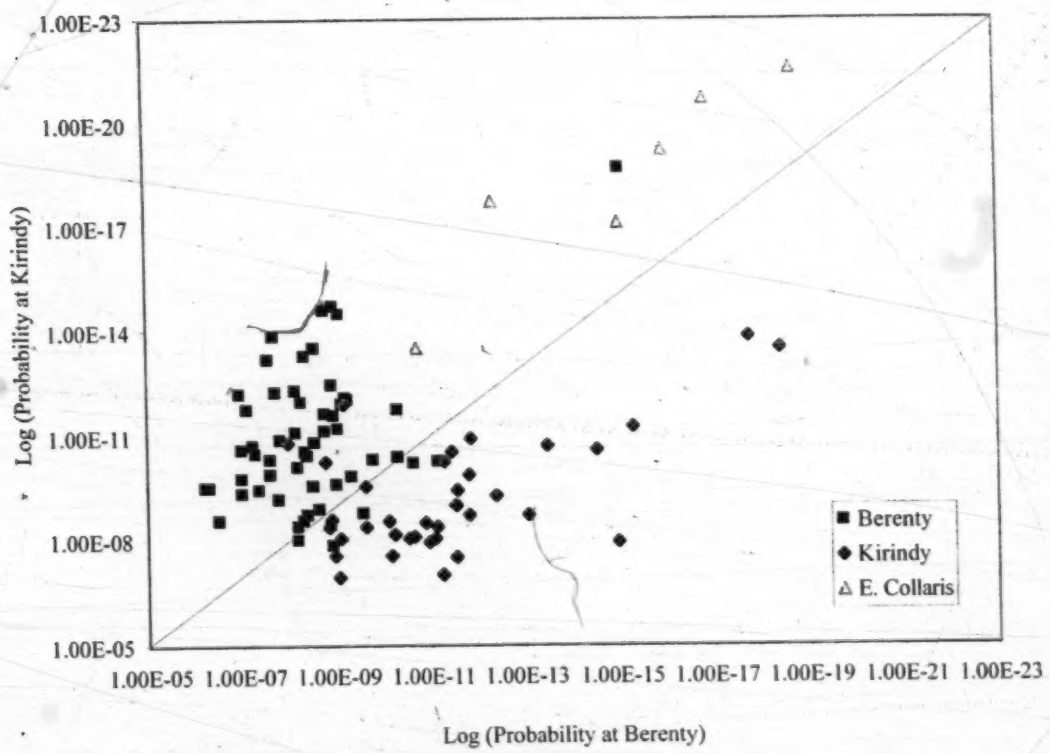

Figure 2-3: Population assignment for individuals from Berenty and Kirindy populations, and for six pure-strain *E. collaris*.

Above the diagonal is the area where individuals assign as more likely being from the Berenty population, and the area below the diagonal does likewise for the Kirindy population. The actual assignment probabilities for individuals from Berenty, Kirindy, and six pure-strain *E. collaris* from natural populations are graphed as squares, diamonds, and triangles, respectively. Assignment probability decreases when moving up and right from the origin along the diagonal.

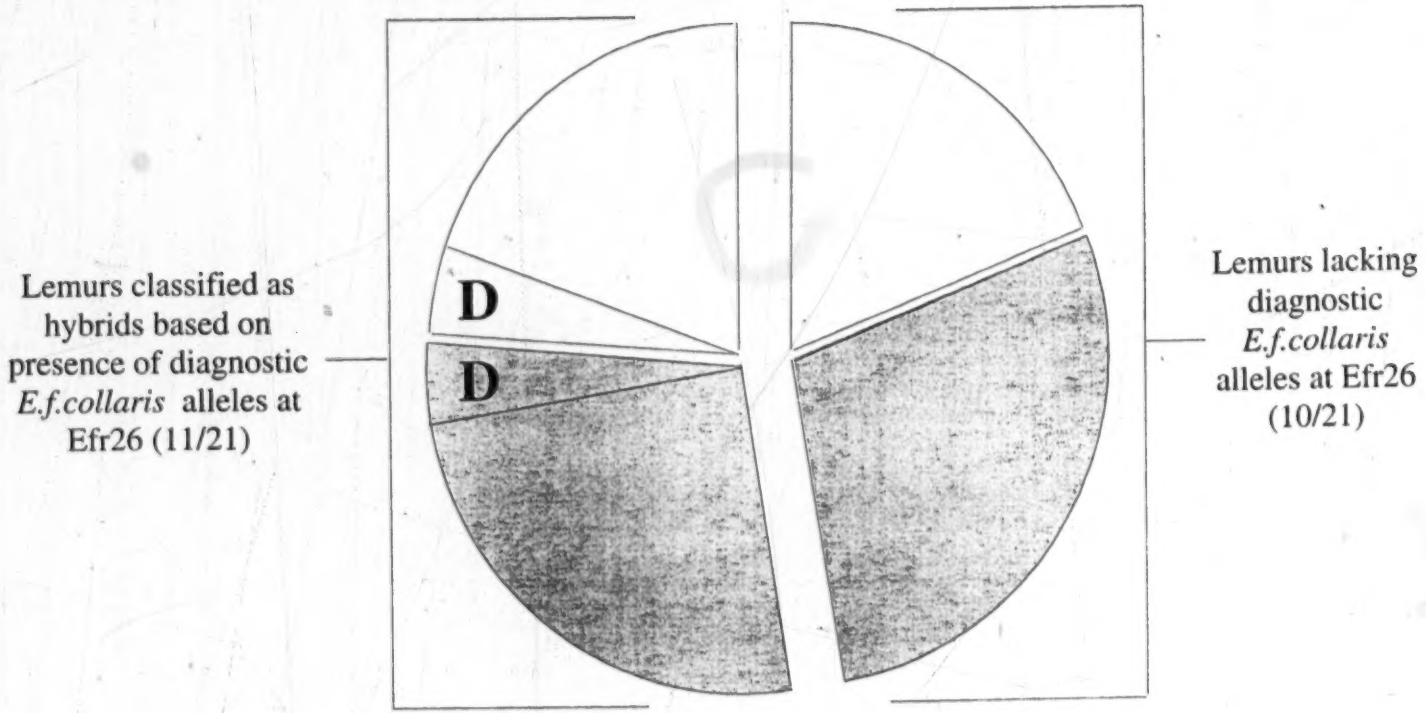
Lemurs classified as hybrids based on presence of diagnostic *E.f.collaris* alleles at Efr26 (11/21)
D
D
Lemurs lacking diagnostic *E.f.collaris* alleles at Efr26 (10/21)

Figure 2-4: Classification of 21 hybrid lemurs in the Berenty population (1996 sample), using only the phylogenetic diagnostic marker, locus Efr26.

Darker regions represent 9 animals classified as hybrids based on morphology, while lighter regions represent the 12 individuals that were not classified as hybrids based on morphology. The "D" areas are 2 individuals that are homozygotes for *E. collaris* alleles.

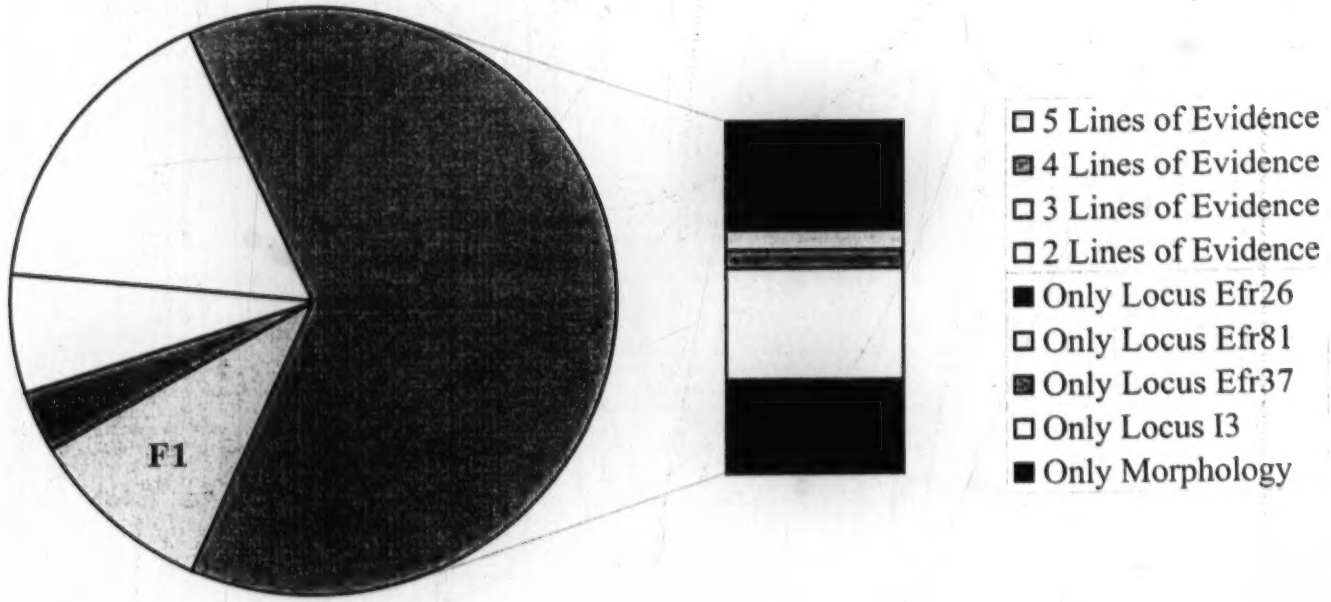

Figure 2-5: Depiction of the number and types of lines of evidence used to determine the status of the 30 hybrid lemurs at Berenty Reserve.

The five lines of evidence used were Locus Efr26, Locus Efr81, Locus Efr37, Locus I3, and Morphological Determinants. The 63% of individuals that were determined by only a single line of evidence are further subdivided on the adjacent stacked bar, described in the lower portion of the legend. The "F1" denotes three individuals that are heterozygotes for all loci diagnostic of hybridization, as well as hybrids based on morphology, likely first-generation hybrids.

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Chapter 3: Discussion of Future Directions

Further research directions with the Berenty population

Several avenues of further research could proceed from this study of brown lemur hybridization at Berenty Reserve. First of all, being able to assess genetic variation in a representative population of *E. collaris* would allow for a confirmation of the suitability of the microsatellite loci for assessing hybridization, as well as allow for comparison of values of genetic variability between the two species. Secondly, cloning and sequencing additional Berenty brown lemur samples for the diagnostic region in locus Efr26 would strengthen the population hybridization assay. Thirdly, the addition of further potential diagnostic or private loci (i.e. Wyner et al. 2002), both nuclear and mitochondrial, would allow for a more complete picture of the extent of hybridization in the population. Since the majority of diagnosis of hybrids was based on only one locus or morphology, it is possible that many more individuals would be likewise diagnosed, through the use of additional loci. In particular, the study of mitochondrial variation would also yield information about the direction of hybridization, for example males of one species with females of the other.

Within-population variation, or relatedness, in the Berenty population yields few obvious patterns. Allele-sharing trees of Berenty individuals indicate a lack of genetic structure in the Berenty population (data not shown), confirmed by fact that the population is apparently in Hardy-Weinberg equilibrium. However, this lack of structure is somewhat problematic since the samples examined in this study do not include full troops of individuals. In contrast, in the past red-fronted brown lemurs have been found to have distinct population structure, with males dispersing and females remaining philopatric, based on genetic and behavioural lines of evidence (Merenlender 1993, Overdorff et al. 1999).

In Berenty, the population has grown very rapidly, and over the years successively larger areas of the reserve were found to contain brown lemurs. There is evidence of many incipient troops being started by females (S. Pinkus, UBC, unpublished

data). It is possible that in natural populations where population size is relatively stable, population structure is as it has been previously described, but that in growing populations the structure is less defined. Perhaps the dispersal of females starting new troops in the still growing Berenty population balances the already well documented dispersal of males, making the population appear unstructured. To assess this more closely, a larger sample of the Berenty population would need to be examined, including several adjacent complete troops.

The observation that the two individuals that appear genetically to be *E. collaris* X *E. f. rufus* F1 hybrids are among the most habituated and obviously aggressive animals in the reserve also deserves some further attention, especially since there are apparently high levels of genetic variation in the Berenty population. It is sometimes the case in animal species that dissimilar pairs of individuals produce fitter-than-average offspring, which has resulted in the evolution of breeding behaviours which take advantage of this fact (Amos and Balmford 2001). It is possible that the behavioural and physiological differentiation between *E. collaris* and *E. f. rufus* is not sufficiently great to create any disadvantage in their offspring, but rather confer some sort of an advantage (hybrid vigour), such as increased ability to garner resources through aggression. It is further possible that some sort of behavioural mechanism in brown lemurs encourages outbreeding, even as far as between species, which could account for the apparent high incidence of hybrids in the population. This sort of mechanism could also explain the apparent high level of genetic diversity in the Berenty population, though it would be very difficult to empirically test these hypotheses.

Behavioural directions

The DNA fingerprinting system developed in this study could help behavioural ecologists elucidate some of the more cryptic elements brown lemur behaviour. For instance, brown lemurs have been found not to exhibit the female dominance characteristic of the related *Lemur catta*, but rather to form more ambiguous "special relationships" in male-female pairs, which in captivity confer only a marginal

reproductive advantage to the males holding the respective relationships (Pereira and McGlynn 1997). To assess the validity of these observations in the wild, one would need to look at the reproductive success of male brown lemurs, for which this DNA fingerprinting system should be ideal. It has also been noted that significant behavioural differences exist between the different brown lemur species and subspecies, pertaining especially to resource partitioning (Tattersall and Sussman 1998). Since brown lemurs exist in both sympatric and allopatric distributions, this DNA fingerprinting system could also be used to help tease apart the effects of habitat and genetic differentiation on the lemurs' resource use, in particular in instances where phylogenetic relationships are unclear, (as in Wyner et al. 1999a).

Conservation genetics directions

At present, all extant lemurs are endangered species, some of them very close to extinction. While ex situ conservation strategies such as keeping zoo populations of many lemur species have begun, such populations are often highly inbred and are likely to represent only a small amount of the genetic diversity found in the wild. For instance, the brown lemur populations kept at Duke University Primate Center (DUPC) have as few as five females being the mothers of all individuals present (Coffman 1994). It is clear that conservation genetics ex situ can be best accomplished through good knowledge of the genetic variation of natural populations, using as many different genetic markers as possible (Scheffrahn et al. 1998), but such information is rarely known among prosimian species (Scheffrahn et al. 2000). Captive breeding and reintroduction programs have been initiated in at least one lemur species, the black and white ruffed lemur, *Varecia variegata variegata* (Wyner et al. 1999b), but the genetic data that was used represents only one mitochondrial locus.

While to my knowledge there are no brown lemur reintroduction efforts currently being organized, the molecular markers used in this study should be valuable for developing the genetic component of a reintroduction strategy, especially when complemented with the additional markers used by Wyner et al. (2002). Further, it will

be possible to use these loci to choose individuals for founding genetically sound captive breeding programs.

Possible future directions beyond brown lemurs

Working on this thesis inspired its expansion into a larger project considering another lemur taxon. Hybridization, in contrast to population fragmentation, has tended to increase genetic variation within individual populations (Dowling and Secor 1997), but to decrease overall diversity among all contributing taxa. Because the effects of human disturbance on genetic diversity are sometimes unclear, population genetic information can be of paramount importance for the development of management strategies for endangered species. Using the genetic screening methods developed in this thesis, it will be possible to study the effects of human-induced hybridization of related taxa and fragmentation of populations on their genetic diversity. The genetic methods also allow for examination of population genetic structure, and gene flow among populations can also be examined, and all of this information related to endangered species management.

A molecular genetic analysis of brown lemur (*Eulemur fulvus*) and black lemur (*Eulemur macaco*) populations, both endangered species threatened increasingly by "slash and burn" agriculture, could be useful. Both of these taxa are species complexes where subspecific differentiation has occurred (Mittermeier et al. 1994). Within each species, hybridization between subspecies occurs in the wild, but does not appear to have negative reproductive consequences when the animals are bred in a captive setting (Rumpler 1975, Rabarivola et al. 1991). Both species are negatively affected by deforestation and other human habitat use; in both species there are some relatively continuous populations, as well as many populations that have been fragmented. Two case studies are described below.

Brown lemurs: In Berenty, a small, isolated reserve in southern Madagascar, introduced red-fronted brown lemurs and collared brown lemurs have hybridized extensively. This population was founded by fewer than ten individuals from each of the two subspecies with non-overlapping ranges. It has since grown to more than 300

individuals. One population has already been compared with the Berenty population, a population near and continuous with the source population of the Berenty red-fronted brown lemurs, in Kirindy Forest, south-eastern Madagascar. It would be possible to sample for comparison the actual source region, Analabe Private Reserve, with permission from the owner of Berenty Reserve.

Within the range of collared brown lemurs, there are both fragmented and continuous populations of animals. The source of the Berenty collared lemurs is in south-eastern rainforest, in one or both of two possible regions: Andohahela, a nature reserve with continuous populations of these animals, and Manangotry, an adjacent heavily settled region of fragmented habitat, hosting several small, insular populations. Samples from collared lemurs from both of these regions could be supplied by Jorg Ganzhorn of Hamburg University, who has done work on a variety of species in these areas. This sort of sampling would allow an examination of the genetic effects of habitat fragmentation in these eastern brown lemur populations, and allow for comparison of their population genetics with Berenty and western populations.

Black lemurs: In north-eastern Madagascar, a team of researchers from the University of Strasbourg, headed by Yves Rumpler, has been studying another species that would be suitable to examine, *Eulemur macaco*. Black lemurs occur as two subspecies. Common black lemurs (*E. m. macaco*) occur on the peninsula of Ambato, and blue-eyed lemurs (*E. m. flavifrons*) are found south of the Andranomalaza river. These two subspecies occur sympatrically. and between the aforementioned two regions, in Sambirano, there is a hybrid zone (Rabarivola et al. 1991). Some common black lemurs also occur on two islands, one a continuous reserve, and the other a settled fragmented habitat. The population genetics of some of these populations have already been studied using RAPD markers (Rabarivola et al. 1998). Human land use has decreased the ranges where each black lemur subspecies occurs alone; the hybrid zone appears to contain more of the total population of black lemurs than it may have historically. How the hybrid zone affects the overall genetic diversity in black lemurs in the region is of interest because of the potential management implications in a region where a national park is currently being developed (Scheffrahn et al. 1998). Genetic diversity in both subspecies, the hybrid zone, and among the mainland and island

populations, could be compared. Furthermore, results of the RAPD analyses that have already been conducted could be compared to microsatellite results, to assess their viability as a population genetic analysis tool in this species.

In order to study the population genetics of brown and black lemurs, it is likely that 30 representative samples from each population considered will suffice. Since most of the microsatellites developed for brown lemurs are also suitable for examining genetic variation in black lemurs, they can be used in both taxa to determine individual genotypes. Furthermore, sequence variation in a region of the mitochondrial genome that is highly variable in these species could be used (as in Wyner et al. 2002). In both the black and brown lemur case studies, one might expect that microsatellite variation would be higher in continuous habitat than in fragmented habitat. It is unclear whether variation in the hybrid Berenty population should be greater or less than that found in pure populations of either subspecies, because there is likely both hybridization and a founder effect present, each of which should pull levels of variation in opposite directions. However, an opportunity now exists to study the genetic effects of a founder effect similar to what happened at Berenty, except with a single subspecies, *E. fulvus mayottensis*. This subspecies has been recently introduced to a small island with a habitat similar to that of Berenty (Gresse et al. 2002), and taking genetic samples from the founding population would allow for tracing the resultant changes in genetic diversity.

The molecular techniques developed as part of this thesis could be used to screen the prospective hybrid zone of black lemurs for hybrids; variation in the hybrid zone will likely be higher than in areas where each subspecies occurs alone on the mainland. A comparison of genetic diversity between the small and large island populations of common black lemurs should elucidate whether or not there is significant gene flow between populations on the large island. The resulting information could then be used to develop management and captive breeding strategies, particularly for black lemurs, the more endangered of the two species.

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ADDENDUM: Characterization of polymorphic brown lemur (*Eulemur fulvus*) microsatellite loci and their amplification in the family Lemuridae*

* A version of this paper was published as Jekielek, J. and Strobeck, C. (1999). Characterization of polymorphic brown lemur (*Eulemur fulvus*) microsatellite loci and their amplification in the family Lemuridae. *Molecular Ecology* 8(5):901-3.

Lemurs are prosimian primates endemic to Madagascar. All extant lemur taxa are considered threatened as a direct result of human-induced habitat degradation and fragmentation, with potential genetic repercussions (Mills & Allendorf 1996). Fine-scale genetic variation has in the past been quantified in lemurs using techniques such as allozymes (Tomiuk *et al.* 1997), with amounts of variation too low to obtain unique individual genotypes, and RAPDs (i.e. Rabarivola 1998), with questionable reproducibility of results (Perez *et al.* 1998). Identification of genetically unique populations for management purposes (Scheffrahn 1998), development of genetically sound breeding programs, and better understanding of lemur social structure are among conservation-related reasons for interest in quantifying lemur genetic diversity. Here, we describe the development of 12 microsatellite loci in brown lemurs (*Eulemur fulvus*) and test them for amplification and variation in 10 species or subspecies of the family Lemuridae.

Several small insert (200-500 bp) red-fronted lemur (*Eulemur fulvus rufus*) genomic libraries were created to isolate microsatellites. Approximately 30µg of genomic DNA was isolated from blood using a standard phenol-chloroform extraction protocol, and digested to completion with Sau3AI (Gibco BRL). Small insert fragments were excised from a 1% agarose gel, purified using QIAquick™ spin columns (QIAGEN), and ligated into BamHI digested dephosphorylated M13mp18RF vector (Gibco BRL). Phages were transfected into *E.coli* DH5∂ F' by either of two methods: the 18°C procedure (Inoue *et al.*, 1990) and electroporation (E. coli Pulser™, Bio-Rad). Over 20 000 recombinant clones, were screened for microsatellites using four biotinylated oligonucleotide probes: $(GT)_{11}$, $(AAGG)_4$, $(GC)_6$, $(GA)_7$. Approximately 200 positive clones were identified using the BluGENE™ nonradioactive detection kit (Gibco BRL).

Inserts from 36 of the strongest positive clone inserts were amplified as in Zheng *et al.* (1995). PCR products were isolated via QIAquick™ spin columns (QIAGEN), and sequenced on an ABI 377 DNA Sequencer using a dRhodamine Terminator Cycle Sequencing kit (Perkin-Elmer). Primer sets were designed using OLIGO software (National Biosciences, Version 5.1) for 21 sequences containing microsatellites; one primer per set was pigtailed to promote preferential amplification of adenylated PCR product (Brownstein *et al.*, 1995), to aid in correct identification of genotypes. The primers were synthesized on a model 391 DNA synthesizer (Applied Biosystems), with one primer of each set flourescently labeled with 6-HEX, 6-TET, or 6-FAM dyes (Perkin Elmer).

To assess amplification and preliminary variability of these prospective microsatellite loci, genomic DNA was isolated from 5-100 plucked hairs from each of 5 brown lemurs (*Eulemur fulvus*), using the QIAamp™ spin column tissue protocol (QIAGEN). Twenty-five µl PCR reactions were conducted, each containing 17µl of the resulting 200µl DNA extraction, 0.16 µM (4 pmols) of each primer, 120 µM dNTP's, 2 µM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (isolated as in Engelke *et al.* 1990) and 10 µM Tris-Cl pH 8.8, 0.1% Triton X100, 50µM KCl and 0.16 mg/ml BSA. Reactions were placed into a 9600 thermal cycler (Perkin Elmer) heated to 94°C, with the following cycling conditions: 1 min at 94°C, three cycles of 30 s at 94°C, 20 s at 54°C, and 5 s at 72°C, followed by 33 cycles of 15 s at 94°C, 20 s at 54°C, and 1 s at 72°C, followed by a final extension of 72°C for 30 s. PCR products were run with internal lane standard on a model 377 DNA Sequencer, and product sizes quantified using Genescan and Genotyper software (Applied Biosystems).

Of the loci tested, 12 yielded easily quantifiable genotypes. To examine the genetic variation at these microsatellite loci, genotypes were obtained for 33 individuals from a closed population of brown lemurs at Berenty Reserve, Madagascar. These results are summarized in Table 1. One locus (Efr26), while exhibiting little size variation, proved to contain considerable intraspecific sequence variation in the regions flanking the microsatellite. All the loci were tested for amplification and variation in 10 taxa from the family Lemuridae, with PCR conditions identical to those above. The results of these cross-species amplifications are summarized in Table 2.

Acknowledgments

Brown lemur genomic DNA for microsatellite development was obtained from the Assiniboine Park Zoo, Winnepeg, Canada. Corey Davis and Bill Clark provided advice regarding the cloning process. Lemur samples from Madagascar were imported under a collaborative accord between the University of Antananarivo, Madagascar and Princeton University, USA. Most samples for cross-species testing were obtained from the Tsimbazaza Zoo, Antananarivo, Madagascar; collared lemur samples were gathered by Tsirivelo Ratovoson in the Manangotry region of southern Madagascar. The Strobeck laboratory is funded in part by Parks Canada and NSERC.

Table A-1: Characteristics of Eulemur fulvus microsatellites (n = 33 individuals).

Underlined primer sequences are pigtails (Brownstein et al., 1995), added to promote adenylated PCR product.

Locus	Size range	Repeat type (and length)	# of alleles	H_O	H_e	Primer sequences (5' to 3')	Accession Number
Efr02	118-122	$(TG)_{14}$	3	0.212	0.449	AAT GTG TAA AAA TTC CTT CT GTG TCC TGG GTG ACA GAG TAA G	AF104088
Efr05	118-151	$(TG)_{15}$	9	0.818	0.735	TGT CTC TGT CTT TCC TAC T GTG TAA AAT GTC TTC TGT ATT CA	AF104089
Efr08	112-160	$(AT)_{16}GTAT(GT)_9$	13	0.756	0.839	AAA GAC AAG GGG TAT G GTT TGC ATT TGA GGA ATC A	AF104090
Efr09	91-105	$(GT)_{15}$	6	0.394	0.788	AAT TTC AGG TAT GCG TGT GTT TGG GGC AAG TTG AAT AG	AF104091
Efr24	92-101	$(GT)_{17}$	8	0.697	0.814	CCC AAT CTC CCT GAG TTT GAG GTT TAG CCT GGG CAA CAT AGT GAG	AF104092
Efr26	252-253	$(GT)_4(GC)_2(GT)_3$	2	0.091	0.088	AGG GAT GTT TGG GAA CTC TG GTT TGG CAG GCT AGG CTG TCA G	AF104093
Efr30	309-325	$(TG)_{19}$	5	0.606	0.637	GAC TAC AAC TTT TCC ATC TG GTG TCA TTG CTG ATT ATT TCT GAT	AF104094
Efr37	219-235	$(TG)_3(AT)_2(GT)_9$	8	0.788	0.760	CCA CAT TTA CTA CAG CAC TAT C GTT TAT TTC TCT ATC CAG GGT TG	AF104095
Efr53	170	$(GAT)_4GACCAT(GAT)_3$	1	0	0	GCA TGT AAG GCA CCT AGC A GTT TAT CTC CTG ACT AAT TGG CAC A	AF104097
Efr56	241-251	$(GTGC)_2(GT)_{16}$	5	0.727	0.704	CCA CCT TAG CAT ATT TAG CAT GTT TGA TGT TCG GAA CTG AGA G	AF104098
Efr81	182-196	$(CT)_3(GT)_{11}$	5	0.636	0.761	TCG TTA TTG AGC ATA TTC A GTG TCG GTT CTT TAC CAA TG	AF104099
Efr88	96-106	$(TG)_{14}$	5	0.8181	0.703	ATC TAA ACC ACT CAG CTA TAA AG GTT TGA TCC CAG GAG AGC TTT	AF104100

Table A-2: Cross-species amplification of 12 brown lemur microsatellite loci.

PCR product size ranges at each locus are given, followed by the number of different alleles in brackets. Absence of PCR product is expressed as a "-"; a multi-banding pattern is denoted by an "M".

	Red-fronted lemur *Eulemur fulvus rufus* N=8	Collared lemur *Eulemur fulvus collaris* N=6	Black lemur *Eulemur macaco macaco* N=4	Blue-eyed lemur *Eulemur macaco flavifrons* N=4	Crowned lemur *Eulemur coronatus* N=4	Red-bellied lemur *Eulemur rubiventer* N=4	Black & White Ruffed lemur *Varecia variegata* N=4	Golden Bamboo lemur *Hapalemur aureus* N=3	Greater Bamboo lemur *Hapalemur simus* N=2	Ring-tailed lemur *Lemur catta* N=1
Efr02	116-126(5)	118-122(2)	116-120(3)	118-122(3)	118(1)	106(1)	116-132(6)	M	125-141(3)	126-128(2)
Efr05	118-151(7)	122-126(3)	114-128(3)	114-126(4)	118-122(4)	118-122(3)	118(1)	118-135(3)	114(1)	116(1)
Efr08	111-137(10)	121-137(6)	97(1)	97(1)	124-130(4)	M	-	101(1)	101(1)	99(1)
Efr09	91-101(3)	95-101(4)	93-99(3)	93-101(4)	99-103(3)	108-112(3)	105-107(2)	97-116(4)	M	91-101(2)
Efr24	94-121(6)	98-104(3)	90-96(4)	90-94(3)	79-90(3)	84-94(3)	M	M	M	-
Efr26	251-253(2)	252-253(2)	252(1)	252(1)	252(1)	251-252(2)	252-263(2)	252-253(2)	252-253(2)	252(1)
Efr30	309-330(8)	309-313(3)	305-315(4)	305-313(4)	307-312(3)	305-313(4)	308-320(2)	305(1)	294(1)	292(1)
Efr37	221-237(7)	225-234(4)	223(1)	223(1)	206-214(2)	-	212(1)	234(1)	223-228(2)	-
Efr53	170(1)	170(1)	166-169(2)	166-169(2)	169-173(2)	168-169(2)	165(1)	169-172(2)	175(1)	175(1)
Efr56	235-251(6)	249-251(2)	243-255(3)	245-255(3)	247-251(3)	245(1)	235(1)	235-237(2)	233(1)	233(1)
Efr81	182-194(6)	182(1)	189(1)	189(1)	190-196(3)	193-201(4)	-	183(1)	185(1)	183(1)
Efr88	98-106(5)	102-106(3)	84-107(6)	94-110(5)	88(1)	88-107(2)	90(1)	94-102(2)	88-107(3)	90(1)

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APPENDIX 1: Morphological hybrid identification*

* adapted from instructions sent to fieldworkers working in the fall of 1997.

E. f. rufus males are identified by:

i) Almost wholly reddish cap, with possibly a small black stripe through its center

ii) Pronounced black stripe through the center of the forehead.

iii) White-gray to slightly reddish sideburns.

iv) Large white eyebrows.

v) Grey body colour.

E. f. collaris males are identified by:

i) Almost completely black face and cap, with often some grey in the eyebrows.

ii) Reddish sideburns (larger than *rufus*).

iii) Blackish-brown body colour.

Hybrid males are identified by:

i) Some red but much black in the cap (black particularly at the back of the cap).

ii) Reddish sideburns (larger than *rufus*).

iii) Some brown in body colour.

E. f. rufus females are identified by:

i) Very large white eyebrows.

ii) Pronounced black stripe in center of forehead

iii) Black or brownish-black cap.

iv) Brown body colour.

No *E. f. collaris* females have been discovered at Berenty.

Hybrid females are identified by:

i) Absence of or a less pronounced stripe on the forehead.

ii) Face and cap of similar colour, grey.

APPENDIX 2: Collaborative Agreement

AGREEMENT

BETWEEN

Barbara Wimmer, Ludwig-Maximillians-University of Munich / German Primate Center Goettingen, Germany

&

Jan Jekielek, University of Alberta, Canada

For the purposes of the project described below, samples used will remain property of the party which conducted the sampling, unless specific agreements are made to the contrary. In all resulting publications where J. Jekielek will be author, Dr. Curtis Strobeck will also be author.

The dataset in question consists of microsatellite genotypes for 31 individuals from the brown lemur population at the C. F. P. F. Forestry Concession locally known as the Kirindy forest. This data was generated between December 10, 1998, and December 20, 1998, at the Ludwig-Maximillians-University of Munich. Both parties agree to share all available information regarding the accuracy of the data, for a period no longer than two years after the signing of this agreement.

It is agreed that these data will be used by J. Jekielek to study hybridization and population genetics of *Eulemur* species, as part of his Ph.D. thesis, and he will retain primary authorship, at his discretion, on all papers resulting from the work he does in this respect. It is agreed that these data will be used by B. Wimmer to study social structure of group-living lemurs, as part of her Ph.D. thesis, and she will retain primary authorship, at her discretion, on all papers resulting from the work she does in this respect. All publications generated during a period no longer than two years after the signing of this agreement will be made available to all parties prior to their submission, to determine potential conflicts regarding data use. If the topic area of such publications is ambiguous, coauthorship rights will be offered to both parties.

Signed,

_____ _____

Barbara Wimmer **Jan Jekielek**

Dated this 18th day of December, 1998, in Munich, Germany